ARCHIVAL REPORT

A Population-Specific Uncommon Variant in *GRIN3A* Associated with Schizophrenia

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Background: Genome-wide association studies have successfully identified several common variants showing robust association with schizophrenia. However, individually, these variants only produce a weak effect. To identify genetic variants with larger effect sizes, increasing attention is now being paid to uncommon and rare variants.

Methods: From the 1000 Genomes Project data, we selected 47 candidate single nucleotide variants (SNVs), which were: 1) uncommon (minor allele frequency <5%); 2) Asian-specific; 3) missense, nonsense, or splice site variants predicted to be damaging; and 4) located in candidate genes for schizophrenia and bipolar disorder. We examined their association with schizophrenia, using a Japanese case-control cohort (2012 cases and 2781 control subjects). Additional meta-analysis was performed using genotyping data from independent Han-Chinese case-control (333 cases and 369 control subjects) and family samples (9 trios and 284 quads).

Results: We identified disease association of a missense variant in *GRIN3A* (p.R480G, rs149729514, $p = .00042$, odds ratio [OR] = 1.58), encoding a subunit of the *N*-methyl-D-aspartate type glutamate receptor, with study-wide significance (threshold $p = .0012$). This association was supported by meta-analysis (combined $p = 3.3 \times 10^{-5}$, OR = 1.61). Nominally significant association was observed in missense variants from *FAAH*, *DNMT1*, *MYO18B,* and *CFB,* with ORs of risk alleles ranging from 1.41 to 2.35.

Conclusions: The identified SNVs, particularly the *GRIN3A* R480G variant, are good candidates for further replication studies and functional evaluation. The results of this study indicate that association analyses focusing on uncommon and rare SNVs are a promising way to discover risk variants with larger effects.

Key Words: Bipolar disorder, *CFB*, *DNMT1*, *FAAH*, *GRIN3A*, *MYO18B*, NR3A, rare variants

Genome-wide association studies (GWAS), which typically examine millions of common (minor allele frequency [MAF] >5%) single nucleotide polymorphisms (SNPs), have been highly successful in identifying genetic variants reproducibly associated with complex human traits (1). Several large-scale GWAS for schizophrenia have been conducted (2–8) and have provided important findings, for example: 1) the identification of genetic loci such as 6p21-p22.1 (major histocompatibility complex region), 1p21.3 (*MIR137*) and 18q21.2 (*TCF4*), associated with schizophrenia at a genome-wide significance level ($p < 5 \times 10^{-8}$); 2) the proposal of a genetic architecture for schizophrenia, which is most likely extremely polygenic, involving possibly thousands of common SNPs conferring a disease risk; and 3) support for genetic overlap between schizophrenia and bipolar disorder, based on the overall profiles of common SNPs and top hit genes, such as *CACNA1C*.

As is the case for most complex diseases, the risk-conferring common SNPs identified so far only have small effect sizes, with odds ratios (OR) predominately ranging from 1.1 to 1.3. Although a recent study indicated that overall profiles of common SNPs could explain a significant part of the variation in liability to schizophrenia (9), single common SNPs have minimal predictive value and it is uncertain whether they contribute greatly to unraveling the pathogenetic mechanisms that lead to schizophrenia. To discover genetic variants with larger effect sizes, emphasis has shifted toward analyzing uncommon (MAF <5%) or rare (MAF <1% or .1%) single nucleotide variants (SNVs), especially those that directly affect protein coding.

Along with a growing interest in rare variants, recent advances in sequencing technology, particularly the emergence of the next-generation sequencing techniques, have enabled us to obtain large volumes of sequence data in a rapid and cost-effective manner. A number of novel and interesting findings have been reported in pioneering works that utilized next-generation sequencing. In studies of psychiatric and neurodevelopmental diseases, involvement of pathogenic de novo SNVs in intellectual disability, autism spectrum disorder, and schizophrenia has been demonstrated (10–18). In studies of nonpsychiatric complex diseases, for example, sick sinus syndrome (19), inflammatory bowel disease (20), and celiac disease (21), uncommon

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protein-affecting variants with larger effects have been identified by resequencing and fine-mapping analyses of loci detected by GWAS. In a resequencing analysis of candidate genes for impulsivity, an uncommon nonsense variant in *HTR2B* is reported to be associated with psychiatric diseases marked by impulsivity (22). The *HTR2B* nonsense variant reported in that study was exclusive to Finns, highlighting the point that uncommon variants are often population-specific. Therefore, intensive studies using non-Caucasian samples provide an opportunity to discover novel, uncommon risk variants with larger effect sizes that cannot be detected in the Caucasian population, the most extensively investigated population in genetic studies to date.

In addition to analysis of de novo variants, fine mapping of loci previously identified in GWAS, and resequencing of targeted candidate regions, we can now utilize dense catalogs of human genetic variations, including uncommon SNVs created by next-generation sequencing projects, such as the 1000 Genomes Project (23) and the Exome Sequencing Project (24), albeit that the latter was confined to European American and African American samples. Uncommon SNVs in these projects were found in seemingly healthy subjects, contrary to extremely rare variants that are robustly associated with psychiatric disorders, such as autism spectrum disorder and schizophrenia, and have been identified exclusively in case samples. However, these uncommon SNVs could represent another class of variants that go some way to provide an explanation for the as yet undiscovered genes responsible for disease heritability. Association analysis of these variants is another promising strategy for identifying genetic risks for common diseases.

In this study, we selected candidate SNVs from the 1000 Genomes Project data, focusing on uncommon, population-specific, and protein-damaging variants. A total of 47 candidate SNVs satisfied the criteria of being: 1) uncommon; 2) missense, nonsense, or splice site variants predicted to be damaging; 3) Asian-specific; and 4) located in candidate genes for schizophrenia and bipolar disorder. These SNVs were subjected to association analysis using a Japanese case-control cohort, consisting of 2012 patients with schizophrenia and 2781 healthy control subjects. We also performed meta-analysis using genotyping data from an independent Han-Chinese case-control set (results from 333 cases and 369 control subjects, previously reported by another group) and family samples (9 trios and 284 quads, genotyped by our laboratory for this study), to analyze the most significantly associated SNV in a Japanese case-control study.

Methods and Materials

Subjects

We used a Japanese case-control sample, consisting of 2012 unrelated patients with schizophrenia (1111 men, 901 women; mean age 48.1 ± 14.4 years) and 2781 control subjects (1197 men, 1584 women; mean age 43.7 ± 14.4 years). We also used 865 samples from unrelated Japanese patients with bipolar disorder (425 men, 440 women; mean age 50.2 ± 14.4 years; 584 with bipolar I disorder, 276 with bipolar II disorder, and 5 with schizoaffective disorder bipolar type) for additional analysis. Samples from bipolar patients and some of the control participants were recruited through the Collaborative Study of Mood Disorders consortium (25). All participants were of Japanese origin and were recruited from the Hondo area of Japan. Populations in the Hondo area are known to fall into a single genetic cluster (26). In our previous analysis using a subset of the same participants, it was shown that $Pr(K = 1)$ (probability that the number of populations present in the sample = 1 [27]) was larger than .99 (28,29) and λ (genomic control factor [30]) was 1.074 (31). These data indicated a negligible population stratification effect in our Japanese samples. All patients had a consensual diagnosis of schizophrenia, according to DSM-IV criteria, from at least two experienced psychiatrists. All healthy control subjects were psychiatrically screened in unstructured interviews. All control subjects and patients gave informed, written consent to participate in the study after being provided with and receiving an explanation of study protocols and objectives.

We also genotyped Han-Chinese samples from mainland China and Taiwan, consisting of 293 schizophrenia pedigrees (1163 subjects: 9 trios and 284 quads, offspring were all affected) (32). This sample set was collected by the National Institute of Mental Health initiative (http://nimhgenetics.org/). Diagnoses of these samples were made using the Diagnostic Interview for Genetic Studies (33) and the Family Interview for Genetic Studies (34), based on DSM-IV-Text Revision or DSM-IV criteria.

In the meta-analysis, genotyping data collated in a report by Shen *et al.* (35) was included. In that study, case-control samples of Han-Chinese in Taiwan (333 cases and 369 control subjects) were used. All patients in this cohort were diagnosed by senior psychiatrists using the Structured Clinical Interview for DSM-IV-TR Axis I Disorders. Exclusion criteria included psychosis due to general medical conditions, substance-related psychosis, and mood disorder with psychotic features. Mental status of control participants was evaluated by senior psychiatrists in diagnostic interviews.

This study was approved by the ethics committees of all participating institutes.

Selection of Single Nucleotide Variants

We selected candidate SNVs from the 1000 Genome Project data, released on December 16, 2010 (23). In this version, 22,891,767 SNVs with PASS flags for quality controls were included. Among them, 11,573,027 SNVs were detectable in Asians, of which 1,911,710 were Asian-specific. From these, we selected variants using the following criteria: 1) missense or splice site variants; 2) variants located within the 1568 genes registered in SZGene (36) or the Bipolar Disorder Gene Database (37) (accessed in January 2011); 3) variant calls with a Phred score >10, indicating a call accuracy of >90%; 4) variants with MAF <5% in Asians; and 5) variants with MAF >0 and <5% in the Japanese population. A total of 308 variants remained after the selections. These included one variant located at an essential splice site. There were no nonsense variants. To narrow down the list further, we evaluated functionality of the remaining missense variants, using conservation scores from 46 vertebrate species (phyloP score, collected from the University of California Santa Cruz (UCSC) genome browser [38] [University of California, Santa Cruz, California]) and two programs predicting the impact of missense variants (PolyPhen2 [39] and SIFT [40]). Finally, 47 SNVs, comprising one essential splice site variant and 46 putatively functional missense variants (a phyloP score >2, predicted to be probably or possibly damaging by PolyPhen2 and predicted to be damaging by SIFT) were selected. The process of SNV selection is summarized in Table 1.

Genotyping

Genotyping of Japanese case-control samples was performed using the iPlex Assay on the Sequenom MassARRAY platform (SEQUENOM, San Diego, California), according to the

Table 1. Number of SNVs Passing Each Filtering Criterion

Filtering Criteria	Number of SNVs Remaining
With PASS Flag	22,891,767
In Asian	11,573,027
Only in Asian	1,911,710
In Coding Regions (UCSC Genes hg19)	21,679
Nonsense, Missense, or Splice Site	14,305
In SZGene or Bipolar Disorder Gene Database (January 2011, 1568 Genes)	1195
Phred Score >10	392
MAF in Asian <.05	308
MAF in Japanese 0 < and < .05	
phyloP >2 and Damaging in PolyPhen2 and SIFT Prediction or in Essential Splice Sites (or Nonsense)	47 (one splice site and 46 missense variants)

MAF, minor allele frequency; SNV, single nucleotide variant; UCSC, University of California Santa Cruz.

manufacturer's instructions. When primers for the iPlex assay could not be designed or genotyping scatter plots were not well clustered, we first tested the candidate base positions for variant alleles using Sanger sequencing. For this test, we used 384 samples consisting of 128 samples each of patients with schizophrenia, patients with bipolar disorder, and controls subjects. These were a part of the samples described in the Subjects section. Single nucleotide variants validated by this analysis were then genotyped using the TaqMan SNP genotyping assay (Applied Biosystems, Grand Island, New York). To evaluate the genotyping accuracy of the multiplex iPlex Assays, we included two previously analyzed SNPs (rs2279381 and rs3763627 [41] and unpublished data [Kazuo Yamada, MD, PhD, *et al.*]) that had been genotyped using the TaqMan assay. Genotyping of Chinese schizophrenia pedigree samples collected by the National Institute of Mental Health initiative was performed using the TaqMan assay.

Quality Control and Statistical Analyses

Quality control of genotyping data was performed using the following exclusion criteria: 1) samples with a call rate of <80%; 2) SNVs with a call rate of <98%; 3) SNVs showing significant deviation from Hardy-Weinberg equilibrium (HWE) in control samples ($p < .001$); and 4) SNVs showing a significant difference of success rate (that is, the proportion of individuals with successful genotype data) between cases and control subjects ($p < .001$). Allelic association was analyzed using Fisher's exact test. For analysis of the *CACNA1F* S671C variant (rs143938580) located on chromosome X, one allele per male sample and two alleles per female sample were counted. Analyses of deviation from HWE, success rate for genotyping, allelic association, and the transmission disequilibrium test (TDT) were performed using PLINK software (version 1.0.7) (42). McNemar's exact test for TDT was performed using the exact2×2 package for R (43) (http://www.r-project.org/). Meta-analysis of case-control and TDT data sets and evaluation of sample heterogeneity were performed using the case-control and TDT meta-analysis package, catmap for R (44). The *Q* statistic (45) calculated by catmap was used for the assessment of heterogeneity among data sets. Post hoc calculations of statistical power were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (46) with the multiplicative model.

Results

Allelic Association Analyses

The results of Japanese case-control allelic association analyses are shown in Table 2, while detailed information about selected SNVs and the filtering criteria are shown in Table 1. The *GRIN3A* R480G variant (rs149729514) was most significantly associated with schizophrenia ($p = .00042$, OR = 1.58; two-tailed Fisher's exact test). From the number of SNVs whose association could be tested in this study (43 SNVs), we defined a study-wide significance threshold as .0012 (= .05/43). Association of the *GRIN3A* R480G variant ($p = .00042$) was more significant than this study-wide significance threshold. Nominal association was observed for *FAAH* A356V (rs77101686, $p = .010$, OR = .71), *DNMT1* G876R (rs62621087, $p = .031$, OR = 1.53), *MYO18B* E559K (rs117243697, $p = .032$, OR = 2.35), and *CFB* R576H variants (rs117314762, $p = .046$, OR = .7).

From 47 selected SNVs, we observed no variant alleles for three base positions in our population (*ADRA1A* R376H: rs140512348, *FAT1* T3326A: no rs number, and *HDAC9* G32R: rs79608746). One SNV (*DDX39B* R259C: rs145191873) failed quality control, due to a low call rate. Genotypic distribution of the *CD36* P90S variant (rs75326924) in control subjects deviated from HWE, with nominal significance ($p = .010$). Significant difference in the success rate of genotyping was not observed for any SNVs. The consistency between multiplex iPLEX genotyping in this study and TaqMan genotyping in our previous experiments was >99.9% for rs2279381 and 100% for rs3763627, proving the accuracy of genotyping by the iPLEX Assay.

Replication Analysis and Meta-Analysis of the *GRIN3A* R480G Variant

To test the validity of association between *GRIN3A* R480G variant and schizophrenia, we performed replication and meta-analyses, using two sets of data from independent samples.

In 2009, Shen *et al.* (35) resequenced *GRIN3A* using samples from the Han-Chinese population in Taiwan, consisting of 333 patients with schizophrenia and 369 control subjects. They detected the R480G variant as an uncommon SNV, along with a nonsignificant overrepresentation of the G allele (Table 3, $p = .145$, OR = 1.52, one-tailed Fisher's exact test). Although it has been demonstrated that the genetic clusters generated by Japanese and Han-Chinese populations are different from each other (26), MAFs of the *GRIN3A* R480G variant in our Japanese samples and their Han-Chinese samples from Taiwan were similar (2.1% in Japanese control subjects and 1.9% in Han-Chinese control subjects).

We also analyzed Han-Chinese pedigree samples, consisting of 9 trios and 284 quads with affected offspring. We successfully genotyped 565 out of 577 offspring and their parents and observed a nominally significant overtransmission of the G allele (Table 3, $p = .036$, OR = 1.92, one-tailed McNemar's exact test).

Combining the three data sets, meta-analysis further supported association between the R480G variant and schizophrenia (Table 3 and Fig. 1, combined $p = 3.3 \times 10^{-5}$, OR = 1.61). In this meta-analysis, we included all probands from trio and quad samples. This result did not change significantly when we randomly selected one proband from each pedigree (combined $p = 6.5 \times 10^{-5}$, OR = 1.60). As there was no significant heterogeneity among three data sets (*Q* statistic $p = .85$), the fixed-effect model was applied for meta-analysis.

Table 2. Results of the Allelic Association Analysis

Chr	Position	dbSNP ID	First Appeared[a]	Reference	Variant	Gene Symbol	SNV Property	PhyloP	PolyPhen2	SIFT	MAF		*p* Value	OR
											Cases	Control Subjects		
1	19,071,356	rs57227966	Build 129	G	A	*PAX7*	R484H	5.68	Probably damaging	Damaging[b]	.95	1.28	.14	.74
1	46,874,246	rs77101686	Build 131	C	T	*FAAH*	A356V	4.18	Probably damaging	Damaging	2.19	3.06	.010[c]	.71
2	216,248,878	rs147655202	Build 134	C	T	*FN1*	V214M	2.57	Probably damaging	Damaging	1.61	1.51	.74	1.07
3	58,135,711	rs141559684	Build 134	G	A	*FLNB*	A2107T	6.42	Probably damaging	Damaging	.90	.91	1.00	.99
3	58,140,563	rs138327769	Build 134	C	G	*FLNB*	S2258C	4.46	Probably damaging	Damaging	.12	.18	.61	.69
3	170,732,328	rs1800572	Build 89	C	T	*SLC2A2*	V101I	5.86	Probably damaging	Damaging	3.37	2.68	.050	1.27
4	187,531,047	Not registered	/	T	C	*FAT1*	T3326A	4.95	Probably damaging	Damaging	Variant allele was not observed			
5	131,325,201	rs3763118	Build 107	C	T	*ACSL6*	V126M	3.27	Probably damaging	Damaging	.55	.89	.070	.62
5	168,201,351	rs2288792	Build 100	C	T	*SLIT3*	R395Q	6.1	Probably damaging	Damaging	1.00	1.12	.61	.89
6	31,503,104	rs145191873	Build 134	G	A	*DDX39B*	R259C	2.21	Probably damaging	Damaging[b]	QC failed			
6	31,914,306	rs117314762	Build 132	G	A	*CFB*	R576H	2.5	Probably damaging	Damaging	1.27	1.80	.046[c]	.70
6	87,725,674	rs3828741	Build 107	G	A	*HTR1E*	A208T	5.47	Probably damaging	Damaging	1.11	1.11	1.00	1.00
6	123,101,544	rs2279381	Build 100	C	T	*FABP7*	T61M	3.39	Probably damaging	Damaging	2.62	2.76	.70	.95
6	132,910,485	rs80078646	Build 131	T	A	*TAAR5*	D114V	3.36	Probably damaging	Damaging	3.30	4.08	.055	.80
6	135,644,371	rs148000791	Build 134	T	C	*AHI1*	E536G	4.86	Possibly damaging	Damaging	4.29	4.24	.92	1.01
6	152,614,868	rs80265744	Build 131	C	T	*SYNE1*	R480H	2.08	Probably damaging	Damaging[b]	.30	.29	1.00	1.04
6	155,458,738	rs116960376	Build 132	C	T	*TIAM2*	T541M	4.26	Probably damaging	Damaging	4.07	4.68	.16	.86
7	18,535,896	rs79608746	Build 131	G	A	*HDAC9*	G32R	5.76	Possibly damaging	Damaging	Variant allele was not observed			
7	21,892,164	rs150631721	Build 134	C	T	*DNAH11*	A3666V	4.22	Probably damaging	Damaging	.60	.76	.38	.79
7	48,412,084	rs143050255	Build 134	A	T	*ABCA13*	Q3708L	2.63	Probably damaging	Damaging	1.19	1.51	.21	.78
7	80,286,003	rs75326924	Build 131	C	T	*CD36*	P90S	5.03	Probably damaging	Damaging	4.14	4.79	.15	.86
7	99,366,093	rs12721627	Build 121	G	C	*CYP3A4*	T185S	3.28	Possibly damaging	Damaging	1.64	2.09	.13	.78
8	16,853,208	rs3793405	Build 107	C	G	*FGF20*	G116R	6.27	Probably damaging	Damaging	3.33	3.28	.91	1.02
8	22,421,982	rs77246845	Build 107	C	T	*SORBS3*	P255L	2.92	Probably damaging	Damaging	.78	.51	.11	1.54
8	22,426,701	rs3758036	Build 131	C	T	*SORBS3*	P449L	5.49	Probably damaging	Damaging	2.57	2.48	.79	1.04
8	23,301,426	rs150919990	Build 134	G	A	*ENTPD4*	P202S	3.84	Probably damaging	Damaging	.97	1.26	.20	.77
8	26,627,940	rs140512348	Build 134	C	T	*ADRA1A*	R376H	2.74	Probably damaging	Damaging[b]	Variant allele was not observed			
8	133,634,908	rs76147813	Build 131	G	T	*LRRC6*	P288H	2.05	Possibly damaging	Damaging	5.16	4.85	.50	1.07
9	104,433,256	rs149729514	Build 134	G	C	*GRIN3A*	R480G	4.16	Probably damaging	Damaging	3.28	2.11	.00042[c]	1.58
10	55,663,053	rs149478475	Build 134	C	T	*PCDH15*	G1156R	4.72	Probably damaging	Damaging	.43	.47	.88	.90
10	61,840,324	rs74777754	Build 131	C	T	*ANK3*	R593H	4.36	Probably damaging	Damaging[b]	2.45	1.96	.12	1.26
10	121,286,832	rs117042762	Build 132	C	T	*RGS10*	V44M	2.11	Probably damaging	Damaging	1.05	1.34	.22	.78
12	56,495,023	rs2271188	Build 100	G	A	*ERBB3*	R1127H	5.13	Probably damaging	Damaging[b]	1.52	1.81	.30	.84
12	70,932,745	rs138916804	Build 134	G	A	*PTPRB*	P1725L	4.51	Possibly damaging	Damaging	1.20	1.11	.70	1.08
12	123,022,996	rs75373025	Build 131	A	G	*KNTC1*	T121A	3.69	Probably damaging	Damaging[b]	.65	.54	.50	1.20
12	123,028,739	rs61751320	Build 129	A	G	*KNTC1*	T198A	3.91	Probably damaging	Damaging[b]	1.71	2.05	.25	.83
13	99,356,584	rs2274827	Build 100	G	A	*SLC15A1*	R459C	3.23	Possibly damaging	Damaging	2.66	2.72	.90	.98
13	103,343,179	rs140948695	Build 134	G	T	*METTL21C*	A89E	3.9	Probably damaging	Damaging	.28	.24	.69	1.17
17	67,079,352	rs149614799	Build 134	C	T	*ABCA6*	Splice site	5.28	–	–	.87	.82	.82	1.07
18	21,353,474	rs76572574	Build 131	G	C	*LAMA3*	G399A	5.15	Probably damaging	Damaging[b]	2.50	2.30	.54	1.09
18	70,417,304	rs118113391	Build 132	C	T	*NETO1*	V512I	5.8	Possibly damaging	Damaging[b]	2.24	2.20	.94	1.02
19	10,259,654	rs62621087	Build 129	C	T	*DNMT1*	G876R	3.7	Probably damaging	Damaging	1.44	.95	.031[c]	1.53
21	37,417,981	rs145926295	Build 134	C	T	*SETD4*	V209M	3.56	Probably damaging	Damaging	.61	.51	.57	1.19
22	26,166,934	rs117243697	Build 132	G	A	*MYO18B*	E559K	4.15	Probably damaging	Damaging	.42	.18	.032[c]	2.35
22	26,706,697	rs117917851	Build 132	G	A	*SEZ6L*	E526K	3.29	Possibly damaging	Damaging	2.13	2.05	.83	1.04
22	42,294,751	rs17848351	Build 123	G	C	*SREBF2*	V902L	3.94	Possibly damaging	Damaging	1.49	1.69	.46	.88
X	49,079,494	rs143938580	Build 134	G	C	*CACNA1F*	S671C	4.86	Probably damaging	Damaging	1.38	1.54	.62	.89

Chr, chromosome; dbSNP, Single Nucleotide Polymorphism database; MAF, minor allele frequency; OR, odds ratio; QC, quality control; SNV, single nucleotide variant.

[a]The release version of dbSNP in which each variant appeared first. dbSNP later than the Build129 contains data from the 1000 Genomes project.

[b]Prediction with low confidence.

[c]$p < .05$.

Table 3. Results of the Meta-Analysis

	Japanese Case-Control				Han-Chinese (Taiwan) Case-Control				Han-Chinese (Mainland China and Taiwan) Pedigree				Meta-Analysis	
Allele	Cases	Control Subjects	*p* Value	OR [95% CI]	Cases	Control Subjects	*p* Value[a]	OR [95% CI]	Transmitted[b]	Untransmitted[b]	*p* Value[c]	OR [95% CI]	*p*	OR [95% CI]
G	132	117	.00042	1.58 [1.23–2.03]	19	14	.145	1.52 [.76–3.05]	25	13	.036	1.92 [.95–4.09]	3.3×10^{-5}	1.61 [1.28–2.01]
C	3888	5435			647	724			13	25				
MAF	.033	.021			.029	.019			NA	NA				

CI, confidence interval; MAF, minor allele frequency; NA, not applicable; OR, odds ratio.
[a]Calculated by one-tailed Fisher's exact test.
[b]Transmission from heterozygous parent.
[c]Calculated by one-tailed McNemar's exact test.

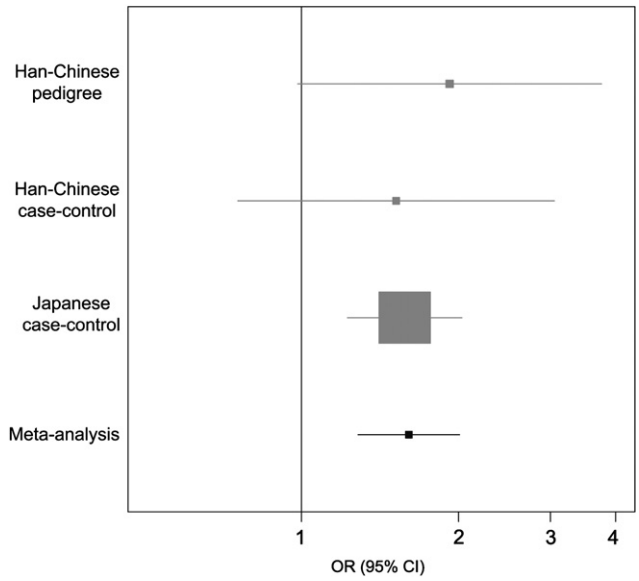

Figure 1. Forest plots of odds ratios (OR) and their 95% confidence intervals (CI) for individual studies and meta-analysis.

Association Analysis of the *GRIN3A* R480G Variant with Bipolar Disorder

There is accumulating evidence to support the existence of overlap in the genetic susceptibility to schizophrenia and bipolar disorder (4,47,48). To test whether *GRIN3A* R480G shows positive association with bipolar disorder, we genotyped samples from Japanese bipolar disorder patients (865 samples). While the G allele, which was overrepresented in schizophrenia, was more frequent in bipolar case samples, we did not observe significant association (p = .138, MAF in cases = 2.72%, OR = 1.3; two-tailed Fisher's exact test). Statistical power obtained from the sample size of bipolar cases and control subjects was 33% for nominal significance (α = .05). Association analysis using combined Japanese schizophrenia and bipolar disorder cases and control subjects (2877 cases and 2781 control subjects) showed study-wide significant association (p = .00077, Bonferroni-corrected p = .033. OR = 1.50), but the association was slightly less significant than that observed with schizophrenia.

Discussion

In this study, we performed association analyses of uncommon, putatively functional, and Asian-specific SNVs with schizophrenia. We observed five significantly associated SNVs with ORs ranging from 1.41 to 2.35. Among them, the *GRIN3A* R480G variant showed the strongest association with study-wide significance (p = .00042, Bonferroni-corrected p = .018). Positive association of the *GRIN3A* R480G variant was observed in independent Han-Chinese pedigree samples (p = .036). Meta-analysis using these data sets and published data from Han-Chinese case-control samples collected in Taiwan further supported this association (combined $p = 3.3 \times 10^{-5}$).

On the other hand, we observed no significant association of the *GRIN3A* R480G variant with bipolar disorder. While calculation of statistical power indicated that the sample size of our bipolar cases was not sufficient to appropriately assess association, it is

possible that this variant may be more relevant to schizophrenia than bipolar disorder.

The *GRIN3A* R480G variant was not detected in Caucasian and African American populations (23,24) and thus far seems Asian-specific. However, if aberrant *GRIN3A* function, driven by genomic variations in this gene, does contribute to the pathogenesis of schizophrenia, other risk variants within this gene could be identified in non-Asian populations (and if so, those variants might be non-Asian specific in some cases).

The association signals in *GRIN3A* have not been detected with genome-wide significance in large-scale GWAS conducted so far. This is not surprising, considering the sample sizes used for the reported GWAS and the MAF and OR of the *GRIN3A* R480G variant observed in this study. For example, if there is a common SNP with 10%, 25%, or 50% MAF in complete linkage disequilibrium (LD) with the R480G variant, the OR of this common SNP would be 1.13, 1.05, and 1.03, respectively, and the number of cases required for 80% power should be 29,012, 83,979, and 248,882, respectively.

Therefore, the sample sizes used in the reported Chinese (7,8) and Japanese (49,50) GWAS have not been sufficient to identify common marker SNPs in LD with the *GRIN3A* R480G variant. Even the largest Caucasian GWAS to date (6) would not have enough power to detect marker SNPs, if other risk-contributing SNVs existed in *GRIN3A* did not generate ORs and/or MAFs much larger than those of the *GRIN3A* R480G variant. This point clearly indicates the advantage of direct genotyping of disease risk-contributing SNVs, especially in studies aiming to identify risk variants with moderate effect sizes, using medium-sized samples.

GRIN3A encodes the NR3A subunit of the *N*-methyl-D-aspartate type glutamate receptor (NMDAR). Involvement of NMDAR hypofunction in the pathophysiology of schizophrenia was first proposed, on the observation that NMDAR antagonists, such as phencyclidine, ketamine, and MK801, induced psychiatric abnormalities that mimic both positive and negative symptoms of schizophrenia (51). The NMDAR hypofunction theory is supported by various lines of evidence from pharmacologic, genetic, postmortem histopathologic, and brain imaging studies (52).

N-methyl-D-aspartate type glutamate receptor is a heteromeric tetramer protein, composed of two obligate NR1 subunits and two subunits from the NR2 or NR3 families. NR3A acts in a dominant-negative manner to suppress NMDAR activity (53,54). NR3A-containing NMDARs display reduced calcium ions (Ca^{2+}) permeability and low sensitivity to magnesium ion (Mg^{2+}) blockade (55,56).

In postmortem brains of patients with schizophrenia, increased *GRIN3A* expression in layer V of the dorsolateral prefrontal cortex (57), along with decreased spine density in the same region (58,59), were reported. Removal of NR3A increases spine density (56) and promotes synapse maturation and memory consolidation (60,61). Male NR3A knockout mice show increased prepulse inhibition, a measure of sensorimotor gating, which is impaired in schizophrenia and other neuropsychiatric disorders (62). These findings collectively suggest the involvement of aberrant, possibly enhanced, *GRIN3A* function in the pathophysiology of schizophrenia, while also implying that the R480G variant of *GRIN3A* promotes a gain of function.

Other significantly associated SNVs are located in *FAAH*, *DNMT1*, *MYO18B*, and *CFB*. *FAAH* encodes the integral membrane enzyme fatty acid amide hydrolase. Another missense variant in this gene (P129T) was reported to be associated with drug addiction (63–65). *DNMT1* encodes DNA (cytosine-5-)-methyltransferase 1, which plays an important role in maintaining methylation patterns in the mammalian genome (66). *MYO18B* encodes an unconventional myosin, myosin-XVIIIb. This gene harbors rs5761163, which is one of the most significantly associated SNPs in GWAS, conducted by the International Schizophrenia Consortium (4). It is noteworthy that the *MYO18B* E559K variant and rs5761163 are in complete LD in the 1000 Genomes Project data. *CFB* encodes complement factor B, a component of the complement system. This gene resides in the major histocompatibility complex class III region on chromosome 6p21.3, a locus associated with schizophrenia, with genome-wide significance (3–6,8). Nevertheless, association of these variants was only nominal and the significant probability of false positivity should be recognized.

To interpret the results of this study more appropriately, two main limitations need to be considered. First, the number of SNVs analyzed in this study was limited. As a selection criterion for SNVs in this study was location in candidate disease genes, it was not surprising to find association of missense variants in promising genes, such as *GRIN3A*. Given the poor replication status of candidate gene association studies for schizophrenia and other psychiatric diseases, more comprehensive and hypothesis-free analyses, particularly genome-wide association studies of uncommon and putatively functional variants are warranted, especially if we are to uncover new genes. Second, the statistical power obtained from our samples was insufficient for testing association of rare and uncommon variants with a stringent threshold of significance. While we applied study-wide significance ($p < .0012$) defined by the number of investigated SNVs in this study, genome-wide significance ($p < 5 \times 10^{-8}$) is needed to demonstrate concrete association. Post hoc statistical power for analysis of the *GRIN3A* R480G variant, calculated from the overall allele counts used in meta-analysis, was 99.3% for nominal significance ($\alpha = .05$) and 88.5% for study-wide significance ($\alpha = .0012$) but only 15.6% for genome-wide significance ($\alpha = 5 \times 10^{-8}$). Moreover, if we consider our Japanese cohort as a sample set for discovery, the statistical power that can be obtained from both sets of Han-Chinese samples for replication are 62.7, 17.0, and .1% for α levels of .05, .0012, and 5×10^{-8}, respectively. To corroborate the association observed in this study, analyses using further independent samples should be conducted.

As that has already been demonstrated in genetic studies of other complex diseases, analysis of uncommon protein-damaging SNVs is a valid method of isolating risk variants with larger effects. The identification of study-wide significant association and the observed ORs of associated SNVs in this study would also support this strategy. More comprehensive genome-wide analysis and replication studies are necessary to overcome the limitations of this study, and they will undoubtedly provide further solid and informative biological insight into the pathophysiology of schizophrenia. This caveat aside, our finding that this putatively functional variant of NR3A, a subunit of the NMDAR, showed significant association with schizophrenia paves the way for functional characterization of this mutation in animal models and sets the stage for the discovery of other uncommon disease-associated variants.

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